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Mechanisms Regulating Adipose-Tissue Pyruvate Dehydrogenase

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1. Isolated rat epididymal fat-cell mitochondria showed an inverse relationship between ATP content and pyruvate dehydrogenase activity consistent with competitive inhibition of pyruvate dehydrogenase kinase by ADP. At constant ATP concentration pyruvate rapidly activated pyruvate dehydrogenase in fat-cell mitochondria, an observation consistent with inhibition of fat-cell pyruvate dehydrogenase kinase by pyruvate. Pyruvate dehydrogenase in fat-cell mitochondria was also activated by nicotinate (100μM) and by extramitochondrial Na^+ (replacing K^+) but not by ouabain or insulin. 2. In rat epididymal fat-pads incubated *in vitro* pyruvate dehydrogenase was activated by addition of insulin in the absence of substrate or in the presence of glucose (10mM) or fructose (10mM). Glucose and fructose activated the dehydrogenase in the absence or in the presence of insulin, and pyruvate also activated in the absence of insulin. It is concluded that extracellular glucose, fructose and pyruvate may activate the dehydrogenase by raising intracellular pyruvate and that insulin may activate the dehydrogenase by some other mechanism. 3. Ouabain (300μM) and medium in which K^+ was replaced by Na^+, activated pyruvate dehydrogenase in epididymal fat-pads. Prostaglandin E_1 (1μg/ml), 5-methylpyrazole-3-carboxylate (10μM) and nicotinate (10μM), which are as effective as insulin as inhibitors of lipolysis and which like insulin lower tissue concentration of cyclic AMP (adenosine 3′:5′-cyclic monophosphate), did not activate pyruvate dehydrogenase. Higher concentrations of prostaglandin E_1 (10μg/ml) and nicotinate (100μM) produced some activation of the dehydrogenase. 4. It is concluded that the activation of pyruvate dehydrogenase by insulin is not due to the antilipolytic effect of the hormone and that the action of insulin in lowering adipose-cell concentrations of cyclic AMP does not afford an obvious explanation for the effect of the hormone on pyruvate dehydrogenase. The possibility that the effects of insulin, ouabain and K^+-free medium may be mediated by Ca^{2+} is discussed.

Insulin treatment of rat epididymal adipose cells activates pyruvate dehydrogenase (EC 1.2.4.1) and this effect of the hormone is antagonized by adrenaline and by adrenocorticotrophic hormone (Jungas, 1970, 1971; Coore *et al.*, 1971). Pyruvate dehydrogenase from adipose cells like the enzyme complex from heart, kidney, liver and brain is inactivated by phosphorylation with $MgATP^{2-}$ and pyruvate dehydrogenase kinase; dephosphorylation leading to reactivation is effected by a Mg^{2+}-dependent pyruvate dehydrogenase phosphate phosphatase (Linn *et al.*, 1969*a*,*b*; Wieland & Siess, 1970; Siess *et al.*, 1971; Coore *et al.*, 1971). Insulin action appears to lead in adipose cells to conversion of pyruvate dehydrogenase phosphate into pyruvate dehydrogenase and thus is likely to involve activation of pyruvate dehydrogenase phosphate phosphatase or inhibition of pyruvate dehydrogenase kinase or a combination of both. On the basis of known mechanisms for the regulation of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase and of known mechanisms for other actions of insulin, adrenaline and adrenocorticotrophic hormone, a number of possible mechanisms for the actions of these hormones on pyruvate dehydrogenase activity require consideration.

Pyruvate dehydrogenase kinase from kidney, heart, brain and liver is inhibited by ADP and by pyruvate (Linn *et al.*, 1969*b*; Wieland & von Jagow-Westermann, 1969; Siess *et al.*, 1971). Evidence has been obtained in the present study that this is also the case for the fat-cell enzyme and for the operation of these mechanisms in fat-cells and fat-cell mitochondria.

The activity of pyruvate dehydrogenase phosphate phosphatase from heart, kidney or adipose-cell mitochondria measured in the presence of Mg^{2+} is markedly increased by low concentrations of Ca^{2+} (Denton *et al.*, 1972). Since ouabain is known to reproduce many of the actions of insulin in adipose cells (Mosinger & Kujalova, 1966; Ho & Jeanrenaud, 1967; Letarte *et al.*, 1969) and might lead to increased intracellular Ca^{2+} concentration (Baker *et al.*, 1969; Baker, 1970; Clausen, 1970), its effects on adipose-

tissue pyruvate dehydrogenase have been investigated.

Insulin may lower and adrenaline and adrenocorticotrophic hormone may increase the concentration of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) in adipose cells and these changes may mediate the inhibitory effects of insulin and the stimulatory effects of adrenaline and adrenocorticotrophic hormone on triglyceride hydrolysis (Butcher *et al.*, 1966; Butcher, 1970; Margariello *et al.*, 1971; Rizack, 1964; Huttenen & Steinberg, 1971). In view of this, it seems pertinent to investigate the possibility that the effect of insulin and its hormonal antagonists on pyruvate dehydrogenase activity in adipose cells may be brought about by changes in either the cyclic AMP concentration or the rate of fatty acid production from triglyceride. In rat heart muscle, perfusion with medium containing fatty acids (also ketone bodies or acetate) has been shown to result in an increase in the inactive phosphorylated form of pyruvate dehydrogenase (Wieland *et al.*, 1971*a*). Moreover, there appears to be an inverse relationship between pyruvate dehydrogenase activity in liver, heart muscle and kidney and plasma free fatty acid concentrations in a number of different metabolic states (Wieland *et al.*, 1971*b*, 1972). We have therefore compared the effects of insulin on triglyceride hydrolysis and pyruvate dehydrogenase activity with those of other agents known to inhibit triglyceride hydrolysis and to lower cyclic AMP concentrations in adipose cells [prostaglandin E_1 (Steinberg *et al.*, 1964; Butcher & Baird, 1968); nicotinate (Carlson, 1963; Butcher *et al.*, 1968); 5-methylpyrazole-3-carboxylate (Froesch, 1967; Butcher, 1970)].

Experimental

Materials

Rats. Epididymal fat-pads were obtained from male albino Wistar rats (150–200g) with free access to a stock laboratory diet (modified 41B; Oxoid Ltd., London, S.E.1, U.K.). The animals were killed by decapitation and in any one experiment were closely matched for age and weight.

Chemicals. These were as given in Coore *et al.* (1971), except as follows. Ouabain (Strophanthin G) was from BDH Chemicals Ltd., Poole, Dorset, U.K. and was diluted into incubation media from a fresh stock solution of 10mM in 80% ethanol. Sodium nicotinate and firefly lantern extract were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. and 5-methylpyrazole-3-carboxylic acid was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.

Media. Fat-pads were incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5).

Isolated fat-cells and fat-cell mitochondria. These were prepared as described by Martin & Denton (1971).

Methods

Extraction and analysis of enzymes in fat-pads and fat-cell mitochondria. At the end of incubation, pads were quickly and lightly blotted and then immediately frozen and ground in liquid N_2. Samples of the frozen powder were extracted by homogenizing with ice-cold 100mM-potassium phosphate buffer, pH7.0, containing 2mM-EDTA. Pyruvate dehydrogenase and glutamate dehydrogenase (EC 1.4.1.2) in the infranatant obtained by centrifugation of the extract at 16000*g* for 2min were assayed at 30°C as described by Coore *et al.* (1971).

At the end of incubation, fat-cell mitochondria were packed by centrifuging at 4°C for 2min at about 16000*g* in an Eppendorf 3200 centrifuge and the pellet was frozen by plunging the plastic centrifuge tube into liquid N_2. Pyruvate dehydrogenase and glutamate dehydrogenase were extracted and assayed at 30°C as described by Coore *et al.* (1971).

Analysis of incubation media. Glucose, lactate and glycerol in neutralized $HClO_4$ extracts of incubation media were measured as described by Coore *et al.* (1971).

Assay of ATP in fat-pads and fat-cell mitochondria. Frozen pad powder (100mg) was extracted with 5% (v/v) $HClO_4$ (2ml) and diethyl ether (10ml) in a motor-driven all-glass homogenizer. After centrifugation, the ether layer was aspirated off and the process repeated with a fresh sample of ether. Ether remaining in a sample of the fat-free extract was removed under vacuum. ATP was assayed in the extract by the luciferin–luciferase method (Stanley & Williams, 1969). For the estimation of ATP in mitochondria, a sample (100μl) of mitochondria and medium was removed at the end of incubation and vigorously mixed with an equal volume of 5% $HClO_4$ by using a vortex mixer. After centrifugation at 16000*g* for 2min ATP was assayed in a sample of the supernatant by the above method.

Expression of results. A unit of enzyme activity refers to the amount of enzyme catalysing the conversion of 1μmol of substrate in 1min at 30°C. The activity of an exclusively mitochondrial enzyme, glutamate dehydrogenase (Martin & Denton, 1970), has been measured as a convenient index of recovery of mitochondrial enzymes from both pads and isolated mitochondria. Its activity has previously been found to be unaffected by incubation under a number of conditions including the presence of insulin (Coore *et al.*, 1971). In the present study, the activity of this enzyme in terms of wet weight of fat-pad was not altered significantly by incubation of the fat-pads under any of the conditions used.

Results

Effects of changes in adenine nucleotide contents on the activity of pyruvate dehydrogenase

Fat-cell mitochondria were incubated under a number of conditions which would be expected to lead to changes in the mitochondrial content of ATP (Table 1). In the absence of added substrate, addition of oligomycin caused the ATP content of fat-cell mitochondria to decrease and this was accompanied by a significant increase in the activity of pyruvate dehydrogenase. Similarly, addition of the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, in the presence of oxoglutarate and malate also lowered the ATP content and resulted in a marked, but somewhat variable increase, in pyruvate dehydrogenase activity. Addition of an oxidizable substrate, either succinate or oxoglutarate with malate, increased the ATP content and decreased the activity of pyruvate dehydrogenase (see also Fig. 2 and Table 2). The only exception we found to this inverse relationship between ATP content and pyruvate dehydrogenase activity was on addition of oligomycin to mitochondria incubated in the presence of oxoglutarate and malate. Here, although the ATP content may have been decreased slightly, the activity of pyruvate dehydrogenase was halved.

The effect of decreasing the concentration of ATP by addition of an uncoupler was also demonstrated with intact fat-pads. Pads were incubated for 30min (see the legend to Table 3), except that fructose (10mM) was added to the preincubation and incubation media. Addition of dinitrophenol (2mM) to the incubation media led to a threefold increase in the activity ratio pyruvate dehydrogenase/glutamate dehydrogenase from 0.097±0.016 to 0.311±0.044 (mean±S.E.M. for four observations in each case).

Effects of pyruvate on the activity of pyruvate dehydrogenase

Fig. 1 shows a time-course of activation of pyruvate dehydrogenase in an extract of fat-cell mitochondria by Mg^{2+} (10mM) followed by inhibition by ATP (1mM). The extract was prepared by freezing and thawing fat-cell mitochondria three times in phosphate buffer, pH7.0, containing oligomycin and mercaptoethanol (Coore *et al.*, 1971). Before addition of ATP the extract was diluted tenfold; at this dilution and Mg^{2+} concentration (1mM) the activity of the phosphatase is greatly impaired.

The rate of decrease of activity on the subsequent addition of ATP was markedly less if pyruvate (1mM) was present; thus phosphorylation of fat-cell pyruvate dehydrogenase appears to be inhibited by pyruvate as shown previously for the enzyme from a number of other mammalian tissues (Linn *et al.*, 1969*b*; Wieland & von Jagow-Westermann, 1969; Siess *et al.*, 1971).

Fig. 2 shows the time-course of the effect of addition of pyruvate on ATP content and the pyruvate dehydrogenase/glutamate dehydrogenase activity ratio in isolated fat-cell mitochondria. In the presence of oxoglutarate (5mM) and malate (0.5mM) the activity ratio pyruvate dehydrogenase/glutamate dehydrogenase decreased in 5min from an initial value of 0.60 to 0.21 and the ATP content increased from 5.3 to about 11nmol/unit of glutamate dehydrogenase. On addition of pyruvate, the activity ratio increased fourfold to 0.81 within 3min. Over this time-period

Table 1. *Activity of pyruvate dehydrogenase in fat-cell mitochondria incubated in the presence of oligomycin or carbonyl cyanide m-chlorophenylhydrazone*

Mitochondria (approx. 250μg of protein) were incubated for 10min at 30°C in KCl medium (1ml) [KCl (125mM), tris–HCl (20mM), EGTA (2mM), $MgCl_2$ (2mM), KH_2PO_4 (2mM), pH7.4] with additions as given in the table. Results are given as means±S.E.M. of the numbers of observations in parentheses. * $P<0.05$; ** $P<0.01$ versus appropriate control.

Additions to incubation media	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	ATP content (nmol/unit of glutamate dehydrogenase)
None (control)	0.34±0.02	8.4±1.1 (3)
Oligomycin (0.5μg/ml)	0.58±0.02**	5.5±0.1* (3)
Oxoglutarate (5mM)+malate (0.5mM) (control)	0.09±0.01	16.1±0.9 (9)
Oxoglutarate (5mM)+malate (0.5mM) +oligomycin (0.5μg/ml)	0.05±0.01**	11.6±2.4 (9)
Oxoglutarate (5mM)+malate (0.5mM) carbonyl cyanide+*m*-chlorophenylhydrazone (0.5μM)	0.36±0.05**	5.8±1.3** (9)

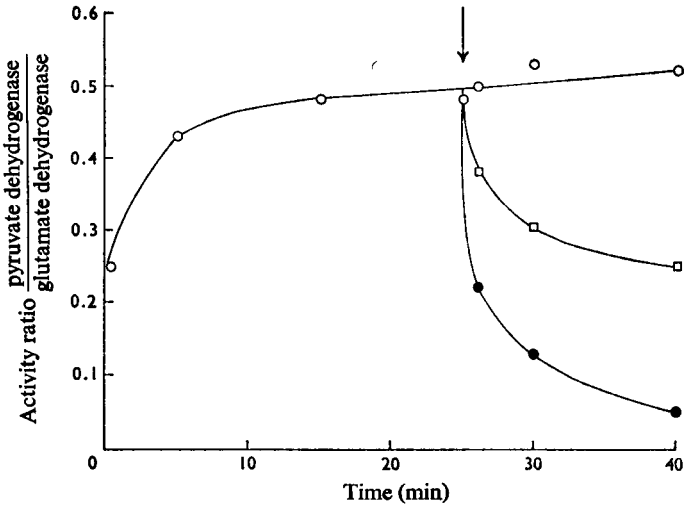

Fig. 1. *Effect of pyruvate on the rate of inactivation by ATP of adipose-tissue pyruvate dehydrogenase in mitochondrial extracts*

Samples of fat-cell mitochondria (approx. 5mg of protein) were extracted by freezing and thawing three times in 0.1ml of 20mM-potassium phosphate buffer, pH7.0, containing oligomycin (0.5μg/ml), mercaptoethanol (5mM) and $MgCl_2$ (0.5mM). The extracts were incubated at 30°C and samples (equivalent to 5–10μl of original extract) were taken at intervals for immediate assay of pyruvate dehydrogenase activity. At zero-time $MgCl_2$ (10mM) was added; after 20min the extract was diluted tenfold by addition of phosphate buffer with the additions as shown above. At 25min (indicated by an arrow) additions were made as follows: □, 1mM-pyruvate and 1mM-ATP; ●, 1mM-ATP; ○, no addition. Points shown are the means of three or four separate observations.

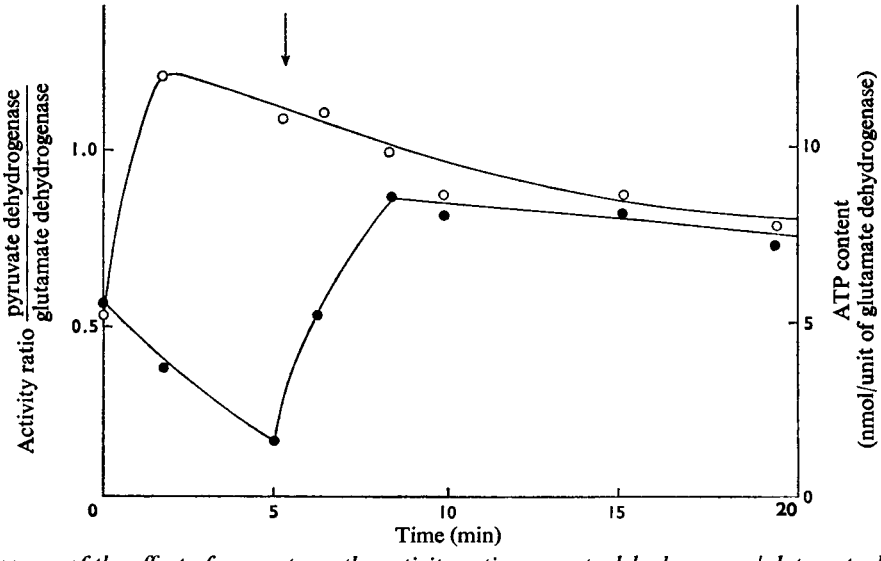

Fig. 2. *Time-course of the effect of pyruvate on the activity ratio pyruvate dehydrogenase/glutamate dehydrogenase* (●) *and ATP content* (○) *in isolated fat-cell mitochondria*

Fat-cell mitochondria (approx. 5mg) were incubated at 30°C in 5ml of KCl medium (125mM-KCl, 2mM-$MgCl_2$, 2mM-EGTA, 2mM-KH_2PO_4, 20mM-tris–HCl, pH7.4) containing oxoglutarate (5mM) and malate (0.5mM). Samples were taken at intervals for extraction and assay of pyruvate dehydrogenase and glutamate dehydrogenase activities and of ATP content as described in the Experimental section. After 5 min (indicated by an arrow) pyruvate (1mM) was added.

Table 2. *Activity of pyruvate dehydrogenase in fat-cell mitochondria incubated in the presence of no substrate, pyruvate, succinate or oxoglutarate and malate*

The procedure is as described in the legend to Table 1. * $P<0.05$; ** $P<0.01$ versus appropriate control.

Expt. no.	Additions to incubation media	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	ATP content (nmol/unit of glutamate dehydrogenase)
1	None (control)	0.453±0.033 (3)	
	Succinate (5mM)	0.238±0.025** (3)	
	Oxoglutarate (5mM)+malate (0.5mM)	0.219±0.013** (3)	
	Pyruvate (10mM)	0.58 ±0.08 (3)	
2	Oxoglutarate (5mM)+malate (0.5mM) (control)	0.111±0.016 (14)	10.2±1.2 (6)
	Oxoglutarate (5mM)+malate (0.5mM) +pyruvate (0.25mM)	0.275±0.075** (6)	10.1±0.6 (6)
	Oxoglutarate (5mM)+malate (0.5mM) +pyruvate (1mM)	0.304±0.01** (7)	16.1±3.3 (3)
3	Succinate (5mM) (control)	0.123±0.012 (3)	
	Succinate (5mM)+pyruvate (0.25mM)	0.152±0.004 (3)	
	Succinate (5mM)+pyruvate (1.0mM)	0.201±0.004* (3)	
	Succinate (5mM)+pyruvate (5mM)	0.208±0.018* (3)	
	Succinate (5mM)+pyruvate (10mM)	0.190±0.002* (3)	

there was little change in the ATP content of the mitochondria. Table 2 summarizes a number of experiments in which the effect of incubating with pyruvate on pyruvate dehydrogenase in isolated fat-cell mitochondria was compared with the effects of incubating with other substrates. Incubation for 10min with either oxoglutarate (5mM) with malate (0.5mM), or succinate (5mM), leads to an activity ratio pyruvate dehydrogenase/glutamate dehydrogenase which is about one-half of the ratio in mitochondria incubated for the same time with no substrate; in contrast incubation with pyruvate (5mM) leads to an increase in the activity ratio of about 50% (Expt. 1). Addition of pyruvate in the presence of either oxoglutarate and malate (Expt. 2) or succinate (Expt. 3) also leads to an increase in pyruvate dehydrogenase activity. These effects could be observed with as little as 0.25mM-pyruvate. ATP content was assayed in Expt. 2 but no significant change was found.

Activation of pyruvate dehydrogenase in intact pads on addition of pyruvate is shown in Table 3. Exposure of pads to pyruvate (10mM) for 30min resulted in a doubling of the activity of pyruvate dehydrogenase (Expt. 1). However, this increase was in fact no greater and may have been less than that seen in pads incubated with either glucose (10mM) or fructose (10mM). An increase in pyruvate dehydrogenase activity in pads incubated with pyruvate has also been observed by Jungas & Taylor (1972). Addition of pyruvate in the presence of fructose did not cause any activation and in the presence of fructose plus insulin may actually have resulted in a slight inhibition (Expt. 2).

Also shown in Table 3 are the effects of incubating intact fat-pads with insulin in the absence of substrate or in the presence of fructose (Expt. 3). Although the activity of pyruvate dehydrogenase was much higher in the presence of fructose, the effect of insulin in percentage terms was approximately the same under both conditions as that found previously (Coore *et al.*, 1971).

Effects of ouabain, K^+-free medium, prostaglandin E_1, 5-methylpyrazole 3-carboxylate and nicotinate on the activity of pyruvate dehydrogenase

The effects of these agents on pyruvate dehydrogenase activity and glycerol output of fat-pads incubated in the presence of fructose have been compared with those of insulin (Tables 4–6). Glycerol output was taken to be a measure of the rate of triglyceride hydrolysis. In these experiments insulin when added was present at 2munits/ml. This high concentration was added to ensure that the maximum response to insulin was being elicited. In fact, we find that appreciable effects of insulin on pyruvate dehydrogenase activity and glucose uptake can be seen at 3 and 10μunits/ml and that the maximum effect on both processes is achieved at about 30–60μunits/ml.

Ouabain when added at 300μM was nearly as effective as insulin in activating pyruvate dehydrogenase (Table 4). No further activation was observed when

Table 3. *Activity of pyruvate dehydrogenase in rat epididymal fat-pads incubated in the presence of glucose, fructose and pyruvate in the presence and absence of insulin*

Fat-pads (in groups of three) were preincubated at 37°C for 30min in bicarbonate-buffered medium containing appropriate substrate and then were transferred to fresh medium containing the same substrate and, where indicated, insulin. Pads were then extracted as described in the Experimental section. Results are given as means ±S.E.M. for the numbers of observations in parentheses. * $P<0.05$; ** $P<0.01$ versus appropriate control. † $P<0.01$ versus pads incubated in fructose without insulin.

Expt. no.	Additions to incubation media: Substrate (10mM)	Additions to incubation media: Insulin (2munits/ml)	Pyruvate dehydrogenase activity (unit/g wet wt. of pad)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase
1	None (control)	−	0.040±0.009 (4)	0.050±0.009 (4)
	Fructose	−	0.132±0.012** (4)	0.177±0.015** (4)
	Glucose	−	0.104±0.007** (4)	0.137±0.003** (4)
	Pyruvate	−	0.095±0.006** (4)	0.137±0.011** (4)
2	Fructose (control)	−	0.145±0.009 (8)	0.164±0.014 (8)
	Fructose+pyruvate	−	0.143±0.009 (8)	0.182±0.010 (8)
	Fructose	+	0.202±0.011** (8)	0.269±0.013** (8)
	Fructose+pyruvate	+	0.179±0.020 (8)	0.218±0.013* (8)
3	None (control)	−	0.028±0.002 (7)	0.036±0.002 (7)
	None	+	0.048±0.005** (7)	0.057±0.004** (7)
	Fructose	−	0.090±0.011** (7)	0.107±0.006** (7)
	Fructose	+	0.166±0.018**† (7)	0.222±0.013**† (7)

both insulin and ouabain were added and in fact the activity with both agents present may have been slightly less than that with insulin alone (Expt. 1). Replacement of medium K^+ ions with Na^+ ions has been shown to result in insulin-like effects on glucose metabolism by adipose cells similar to those of ouabain (Letarte *et al.*, 1969). Consistent with this, the activity of pyruvate dehydrogenase was increased in fat-pads incubated in K^+-free medium (Expt. 2). The increase was perhaps less than that seen with ouabain and was only about one-half that seen with insulin in the same experiment. Glycerol output was decreased by insulin, ouabain and K^+-free medium by 58, 49 and 44% respectively. A few measurements of ATP content were made but no significant changes were observed, in agreement with the previous findings of Bihler & Jeanrenaud (1970) with isolated fat-cells.

Previously, we found that prostaglandin E_1 at 1μg/ml did not reproduce the effect of insulin on pyruvate dehydrogenase activity, although it was as effective as insulin in decreasing triglyceride hydrolysis (Coore *et al.*, 1971). This result has been confirmed and extended in the present study (Table 5). When added at 1μg/ml no change in pyruvate dehydrogenase activity was found whereas the inhibition of glycerol output was nearly identical with that observed with insulin (Expts. 1 and 3). When added at 10μg/ml a stimulation of 20–30% was observed but this increase was only of marginal statistical significance (Expt. 2). Addition of 5-methylpyrazole-3-carboxylate (10μM) also caused no significant change in the activity of pyruvate dehydrogenase whereas it inhibited glycerol output to a similar extent to insulin (Expt. 3).

The effects of nicotinate on pyruvate dehydrogenase activity are shown in Table 6. Although effective at inhibiting glycerol output when added at 1μM, no statistically significant effect on pyruvate dehydrogenase activity was seen at this concentration. However, when added at 100μM the activity of pyruvate dehydrogenase was increased by approx. 60%. In the same experiment the increase with insulin was approx. 110%. Activation of pyruvate dehydrogenase by high concentrations of nicotinate (70μM) has also been found by Jungas & Taylor (1972).

The established actions of ouabain and insulin are on components of the cell membrane so it is perhaps not surprising that when isolated fat-cell mitochondria were incubated with either ouabain or insulin in the presence of oxoglutarate and malate no change in pyruvate dehydrogenase activity or ATP content was detected (Table 7). However, addition of nicotinate (100μM) under the same conditions consistently increased the activity of pyruvate dehydrogenase (the average increase for nine observations was 59%); the ATP content appeared to decrease by about 20% (Expt. 1). Addition of nicotinate (250μM) to mitochondria incubated with oxoglutarate, malate and pyruvate also resulted in a small but significant increase in pyruvate dehydrogenase activity; under

Table 4. *Pyruvate dehydrogenase activity in rat epididymal fat-pads incubated in K^+-free medium and in the presence of ouabain*

Details are as given in the legend to Table 3 except that fructose (10mM) was added to all preincubation and incubation media. For K^+-free medium K^+ ions in the bicarbonate-buffered medium were replaced by equivalent Na^+ ions. * $P<0.05$; ** $P<0.01$ versus appropriate control.

Expt. no.	Additions to incubation media	Pyruvate dehydrogenase activity (units/g)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	Glycerol output (μmol/h per g)	ATP content (nmol/g)
1	None (control)	0.085±0.007 (7)	0.159±0.011 (7)		
	Insulin (2munits/ml)	0.171±0.006** (7)	0.308±0.008** (7)		
	Ouabain (300μM)	0.149±0.008** (7)	0.244±0.005** (7)		
	Insulin (2munits/ml) +ouabain (300μM)	0.147±0.01** (7)	0.257±0.012** (7)		
2	None (control)	0.135±0.004 (8)	0.209±0.014 (8)	2.56±0.35 (4)	135±29 (4)
	Insulin (2munits/ml)	0.225±0.011** (8)	0.388±0.034** (8)	1.07±0.08* (4)	125±25 (4)
	Ouabain (300μM)	0.181±0.011** (8)	0.310±0.029** (8)	1.31±0.34* (4)	101±27 (4)
	K^+-free medium	0.168±0.009** (8)	0.291±0.025** (8)	1.44±0.20* (4)	113±8 (4)

Table 5. *Activity of pyruvate dehydrogenase in rat epididymal fat-pads incubated in the presence of prostaglandin E_1, 5-methylpyrazole-3-carboxylate and insulin*

Details are as given in the legend to Table 3 except that fructose (10mM) was added to all preincubation and incubation media. * $P<0.05$; ** $P<0.01$ versus appropriate control.

Expt. no.	Additions to incubation media	Pyruvate dehydrogenase activity (units/g)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	Glycerol output (μmol/h per g)
1	None (control)	0.160±0.011	0.207±0.014	1.50±0.18 (16)
	Insulin (2munits/ml)	0.235±0.013**	0.311±0.018**	0.76±0.06** (16)
	Prostaglandin E_1 (1μg/ml)	0.164±0.010	0.224±0.013	0.79±0.09** (16)
2	None (control)	0.154±0.010	0.213±0.021	1.36±0.10 (8)
	Insulin (2munits/ml)	0.238±0.10**	0.345±0.021**	0.70±0.10** (8)
	Prostaglandin E_1 (10μg/ml)	0.181±0.016	0.278±0.014*	0.66±0.10** (8)
3	None (control)	0.14 ±0.02	0.18 ±0.03	1.5 ±0.4 (4)
	Insulin (2munits/ml)	0.23 ±0.03*	0.33 ±0.05*	1.0 ±0.2**† (4)
	5-Methylpyrazole-3-carboxylate (10μM)	0.14 ±0.01	0.20 ±0.02	1.0 ±0.2*† (4)
	5-Methylpyrazole-3-carboxylate (10μM)+insulin (2munits/ml)	0.27 ±0.02**	0.35 ±0.02**	1.1 ±0.2**† (4)
	Prostaglandin E_1 (1μg/ml)	0.16 ±0.03	0.22 ±0.04	0.9 ±0.2**† (4)

† Value was calculated by using paired differences.

Table 6. *Effects of nicotinate on pyruvate dehydrogenase activity in rat epididymal fat-pads*

Details are as given in the legend to Table 3 except that fructose (10mM) was added to all preincubation and incubation media. * $P<0.01$ versus control.

Additions to incubation media	Pyruvate dehydrogenase activity (unit/g)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	Glycerol release (μmol/h per g)
None (control)	0.074±0.007	0.116±0.009	1.64±0.20 (6)
Nicotinate (1μM)	0.084±0.007	0.131±0.005	0.74±0.05* (6)
Nicotinate (100μM)	0.111±0.009*	0.185±0.010*	0.83±0.10* (6)
Insulin (2munits/ml)	0.143±0.013*	0.268±0.028*	0.68±0.13* (6)

Table 7. *Activity of pyruvate dehydrogenase in fat-cell mitochondria incubated in the presence of insulin, ouabain, nicotinate or Na^+ ions*

Details are as given in the legend to Table 1 except that the KCl medium was supplemented with oxoglutarate (5mM), malate (0.5mM) and further additions as given in the table. Results are given as means±S.E.M. for the numbers of observations in parentheses. * $P<0.05$; ** $P<0.01$ versus appropriate control.

Expt. no.	Additions to incubation media	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	ATP content (nmol/unit of glutamate dehydrogenase)
1	None (control)	0.132±0.004 (9)	11.7±0.35 (9)
	Insulin (2munits/ml)	0.145±0.011 (9)	11.9±1.28 (6)
	Ouabain (100μM)	0.126±0.005 (7)	11.0±0.80 (7)
	Nicotinate (100μM)	0.210±0.025** (9)	9.8±0.57* (9)
	Na^+ (30mM)†	0.156±0.017 (6)	13.7±1.60 (5)
	Na^+ (70mM)†	0.161±0.017 (6)	13.2±0.59 (3)
	Na^+ (125mM)†	0.167±0.013** (6)	13.1±0.29 (3)
2	Pyruvate (0.25mM) (control)	0.280±0.013 (9)	11.9±0.59 (8)
	Pyruvate (0.25mM), nicotinate (0.25mM)	0.352±0.023* (9)	15.2±2.20 (9)

† In these experiments an equivalent amount of the KCl medium was replaced by the stated concentration of NaCl.

these conditions no decrease in ATP content was apparent. Replacement of some or all of the K^+ in the mitochondrial incubation medium with an equivalent concentration of Na^+ also apparently led to a small increase in pyruvate dehydrogenase activity, but this increase was only statistically significant when all the K^+ ions were replaced by Na^+ ions. ATP content remained unaltered.

Discussion

It seemed to us important that regulatory mechanisms proposed for pyruvate dehydrogenase as a result of studies with the isolated enzyme complex and its components should be shown to operate in the intact mitochondrion. This has been examined in the present study and the extent to which these mechanisms may mediate the actions of insulin and other hormones has been assessed.

Regulation of pyruvate dehydrogenase in fat-cell mitochondria by changes in adenine nucleotide and pyruvate concentrations

Fat-cell mitochondria, like those of liver and other tissues, appear to contain a transport system exchanging ATP for either ATP or ADP (Martin & Denton, 1971; Klingenberg, 1970). In the present study mitochondria were incubated without addition of external nucleotide; the measured ATP concentrations are therefore likely to be intramitochondrial. An inverse relationship between pyruvate dehydrogenase activity and ATP content in fat-cell mitochondria was observed. The mechanism whereby a decrease in mitochondrial ATP content increases pyruvate dehydrogenase activity may involve not only competitive inhibition of pyruvate dehydrogenase kinase by an increased [ADP]/[ATP] ratio (Linn *et al.*, 1969*a*) but also activation of pyruvate dehydrogenase phosphate

phosphatase by Mg^{2+} and Ca^{2+} (these concentrations may increase because the affinity of ADP for these ions is much lower than that of ATP).

Addition of pyruvate to fat-cell mitochondria in which pyruvate dehydrogenase had been inactivated by ATP formed by respiration of 2-oxoglutarate and malate, led to rapid and substantial activation of the dehydrogenase at a constant ATP content. Although pyruvate is an inhibitor of fat-cell pyruvate dehydrogenase kinase, no direct effects of pyruvate on the pig heart phosphatase have been detected (R. M. Denton & P. J. Randle, unpublished work). If this is applicable to the fat-cell mitochondrion then it seems likely that both kinase and phosphatase are active in the absence of pyruvate and that appreciable recycling between phosphorylated and non-phosphorylated forms of pyruvate dehydrogenase is occurring. Any condition which leads to an increase in intracellular pyruvate concentration may therefore result in activation of pyruvate dehydrogenase in epididymal adipose cells. The marked activation of the dehydrogenase in adipose cells by addition of pyruvate or by addition of fructose or glucose in the absence or in the presence of insulin may involve this mechanism.

Mechanisms mediating actions of insulin and adrenaline, adrenocorticotrophic hormone and dibutyryl cyclic AMP

Adenine nucleotides. Insulin is unlikely to activate fat-cell pyruvate dehydrogenase by altering adenine nucleotides, because it is well established that the hormone does not lower the ATP concentration or raise that of ADP (Denton & Randle, 1966; Halperin & Denton, 1969; Bihler & Jeanrenaud, 1970; Saggerson & Greenbaum, 1970). Activation of pyruvate dehydrogenase in pads exposed to adrenaline in the absence of substrate and albumin may be the result of a decrease in the concentration ratio of ATP/ADP seen under these conditions (Hepp *et al.*, 1968; Bihler & Jeanrenaud, 1970; Jungas & Taylor, 1972).

Pyruvate. The possibility that insulin may activate pyruvate dehydrogenase by increasing intracellular pyruvate concentration cannot be excluded with certainty. Reliable measurements of intracellular pyruvate concentration are beyond the capabilities of current techniques, because of the high rate of pyruvate turnover coupled with an adverse ratio of extracellular water/intracellular water (approx. 20:1). Nevertheless, indirect evidence would suggest that insulin does not act solely by increasing intracellular pyruvate concentration. The hormone activates the dehydrogenase in the absence of external substrate in pads from normal rats (Coore *et al.*, 1971; Table 3) or from starved re-fed rats (Jungas, 1971). Under these latter conditions of restricted formation of pyruvate from glycogen, the hormone decreases the combined output of lactate and pyruvate and stimulates fatty acid biosynthesis (Jungas, 1971). It is difficult to visualize mechanisms whereby insulin could sustain an increased intracellular pyruvate concentration in the absence of substrate or enhanced glycogenolysis when conversion of pyruvate into fatty acid was increased. With fructose as substrate, addition of insulin has only small effects on the rate of pyruvate formation but there is marked activation of pyruvate dehydrogenase and of fatty acid biosynthesis from pyruvate; under these conditions there is a net uptake of lactate and pyruvate from the medium leading to lower steady-state extracellular concentrations of lactate and pyruvate (Coore *et al.*, 1971). These observations suggest that insulin activates pyruvate dehydrogenase by a mechanism which may actually lead to a lowering of intracellular pyruvate concentration with fructose as external substrate. Finally it may be noted that adrenaline, which antagonizes the effect of insulin on pyruvate dehydrogenase, increases the outputs of lactate and pyruvate and their extracellular concentrations with glucose as substrate (Coore *et al.*, 1971; Denton *et al.*, 1966; Halperin & Denton, 1969).

Lipolysis and cyclic AMP concentration. There is an inverse relationship between the activity of pyruvate dehydrogenase and the rate of lipolysis in fat-cells exposed to insulin, adrenaline, adrenocorticotrophic hormone, dibutyryl cyclic AMP (6-*N*-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate), ouabain or high concentrations of prostaglandin E_1 (10μg/ml) or nicotinate (100μM) (Coore *et al.*, 1971; Table 4). However, this inverse relationship is not obeyed in fat-cells exposed to a low concentration of prostaglandin E_1 (1μg/ml), or nicotinate (1μM) or to 5-methylpyrazole-3-carboxylate (10μM). At the given concentrations these agents were as potent as insulin in suppressing triglyceride hydrolysis but unlike insulin did not activate pyruvate dehydrogenase. The activation of pyruvate dehydrogenase by insulin is thus unlikely to be caused solely by its anti-lipolytic effect.

Similar considerations may apply to the possible role of adipose-cell cyclic AMP concentrations in the regulation of pyruvate dehydrogenase. Adrenaline, adrenocorticotrophic hormone and dibutyryl cyclic AMP may increase adipose-cell cyclic AMP and inhibit pyruvate dehydrogenase. Insulin may lower cyclic AMP concentration and activate pyruvate dehydrogenase. However, the most marked effects of insulin on cyclic AMP concentrations occur in the presence of adrenaline; in the absence of catecholamines insulin has only a small lowering effect on the cyclic AMP concentration (Kuo & De Renzo, 1969). The effects of insulin on pyruvate dehydrogenase are certainly no less marked in the absence of adrenaline than in its presence (Coore *et al.*, 1971). Moreover,

prostaglandin E_1 (1 μg/ml), nicotinate (1 μM) and 5-methylpyrazole-3-carboxylate (10 μM) produce greater decreases in cyclic AMP concentrations in fat-cells in the presence of adrenaline than does a high concentration of insulin (Butcher, 1970). At these concentrations these agents did not reproduce the effect of insulin on pyruvate dehydrogenase.

In the case of phosphorylase, glycogen synthetase and triglyceride lipase an important point of evidence linking hormonal effects on adenylate cyclase with regulation of these enzymes has been the discovery of a cyclic AMP-sensitive protein kinase. However, in the case of pyruvate dehydrogenase there is no evidence that the activity of pyruvate dehydrogenase kinase or pyruvate dehydrogenase phosphate phosphatase are influenced by cyclic AMP or by cyclic AMP-dependent protein kinase (Coore *et al.*, 1971; R. M. Denton & P. J. Randle, unpublished work). Thus if cyclic AMP concentration mediates the effects of hormones on the activity of pyruvate dehydrogenase in adipose cells, it seems necessary to postulate either that other unknown factors are involved in this regulation, or that the regulation is indirect and brought about by effects of the cyclic nucleotide on intramitochondrial concentrations of known effectors of pyruvate dehydrogenase kinase or pyruvate dehydrogenase phosphate phosphatase.

The activation of pyruvate dehydrogenase seen when intact fat-pads are exposed to 100 μM-nicotinate was also observed when isolated fat-cell mitochondria were incubated in the presence of nicotinate at this concentration. It would seem unlikely that this direct effect of nicotinate on mitochondria involves changes in cyclic AMP. The mechanism involved and particularly its relationships to the mechanism of activation of insulin remain to be explored.

Calcium. Fat-cell pyruvate dehydrogenase phosphate phosphatase is activated by physiological concentrations of Ca^{2+} (Denton *et al.*, 1972). Whether or not variations in intracellular Ca^{2+} concentrations may mediate effects of hormones on pyruvate dehydrogenase is not known. It is, however, possible that the ionic movements resulting from incubation of fat-pads with ouabain or in K^+-free medium may increase intracellular Ca^{2+} and thereby lead to activation of pyruvate dehydrogenase.

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